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ARTICLES

Poly(ethylene glycol) Multiblock Copolymer as a Carrier of Anti-Cancer Drug Doxorubicin

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The synthesis of a novel water-soluble polymer drug carrier system based on biodegradable poly(ethylene glycol) block copolymer is described in this paper. The copolymer consisting of PEG blocks of molecular weight 2000 linked by means of an oligopeptide with amino end groups was prepared by interfacial polycondensation of the diamine and PEG bis(succinimidyl carbonate). The structure of the oligopeptide diamine consisting of glutamic acid and lysine residues was designed as a substrate for cathepsin B, a lysosomal enzyme, which was assumed to be one of the enzymes responsible for the degradation of the polymer carrier in vivo. Each of the oligopeptide blocks incorporated in the carrier contained three carboxylic groups of which some were used for attachment of an anti-cancer drug, doxorubicin (Dox), via a tetrapeptide spacer Gly-Phe-Leu-Gly. This tetrapeptide spacer is susceptible to enzymatic hydrolysis. In vitro release of Dox and the degradation of the polymer chain by cathepsin B as well as preliminary evaluation of in vivo anti-cancer activity of the conjugate are also demonstrated.

INTRODUCTION

Water-soluble synthetic polymers conjugated with a desirable targeting moiety (e.g., an antibody) provide a drug delivery system capable of site-specific delivery. In recent years, such conjugates based on copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA)[1] have been extensively studied as drug carriers facilitating site-specific delivery of immunosuppressants and anti-cancer drugs (*1*–*3*). In these conjugates, the nondegradable poly(HPMA) backbone is modified by biodegradable oligopeptide side chains that are terminated with targeting and/

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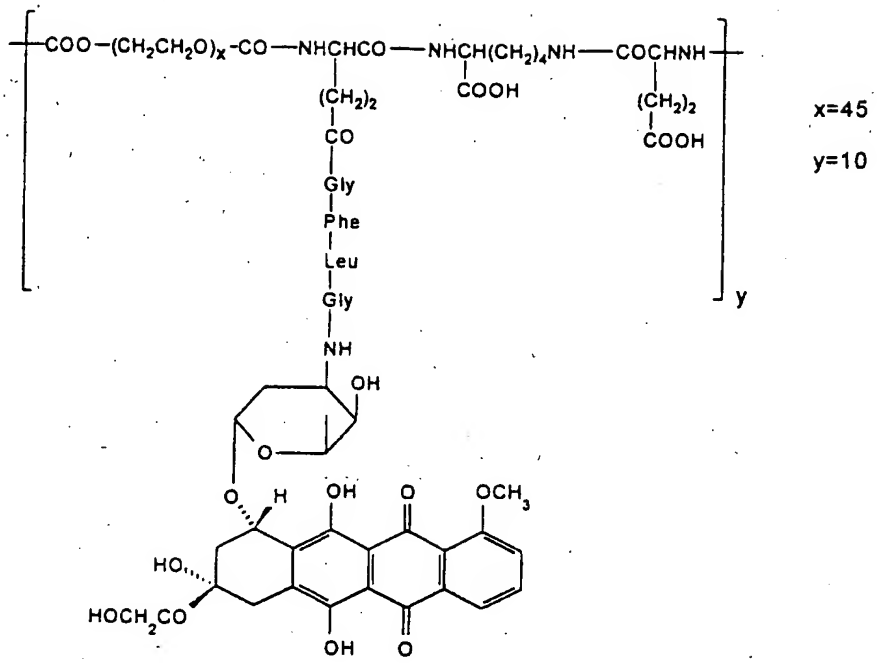
Scheme 1



or drug moieties. The oligopeptide side chains are randomly distributed along the polymer chain and render controlled drug release.

Poly(ethylene glycol) has been the most frequently used hydrophilic polymer for protein modification. It has been found that "pegylated" proteins (enzymes, antibodies, etc.) are more resistant to proteolytic degradation than unmodified ones. They also exhibit much longer circulating times in blood and lower immunogenicity. In some cases, such a modification is necessary for their therapeutic application. Although there were many attempts to use PEG as a drug carrier, its application is rather limited as the molecule of PEG has only two potential binding sites situated at the ends of the polymer chain enabling attachment of only two drug molecules or one drug molecule and one targeting moiety per macromolecule. The molecular weight of some anti-cancer drugs (5-fluorouracil, doxorubicin) (*4*, *5*) modified with single PEG chain was probably still too low to slow their excretion from body and enhance their accumulation in tumors (EPR effect) (*6*), thus improving their therapeutic activity. Zalipsky et al. prepared (*7*) a high-molecular-weight multiblock copolymer PEG-lysine which they also used as a carrier of doxorubicin. Although they reported synthesis of a copolymer with M_w up to 100 000, the nondegradability of the urethane bonds between PEG and Lys residues by proteolytic enzymes still persists as a major drawback of the system.

To overcome this problem, we suggested a high-molecular-weight multiblock PEG-based drug carrier system where PEG blocks are connected by diamine oligopeptide linkages enabling attachment of two or three drug molecules to each repeating block. In our previous studies (*8*, *9*), we have described a model diblock copolymer consisting only of two PEG chains connected by one diamine linkage bearing 4-nitroaniline as a drug model. Using such a model system, we have verified both the degradability of the oligopeptide linkages between the PEG blocks and the possibility to release the drug model from the polymer incubated with lysosomal enzymes. The results of a drug model, 4-nitroaniline, release from polymer carriers based on PEG incubated in cathepsin B solutions, have shown that the relationship between the rate of release and the structure of the oligopeptide spacer is very similar to that found for the poly(HPMA)-based systems (*10*). The conjugates of poly(HPMA) containing doxorubicin linked to the polymer carrier with the oligopeptide spacer Gly-Phe-Leu-Gly exhibit high anti-cancer activity in vivo and are currently being clinically tested. The major drawback of poly(HPMA)-based drug carriers is their nondegradability under physiological conditions. That limits the use of such carriers only to molecular weights lower than renal threshold of the living body (50 000) ensuring safe elimination of the polymer from the organism after releasing the active compound from the carrier. On the contrary, only high-molecular-weight conjugates can be efficiently accumulated in many solid tumors due to the EPR (enhanced permeation and retention) effect (*6*). To obtain a high-molecular-weight carrier facilitating accumulation of drug in a tumor and elimination of the polymer after releasing the cytostatic drug, we have prepared anti-cancer polymer drug based on biodegradable block copolymers of PEG, analogous to poly(HPMA) conjugates, with cytostatic drug doxorubicin (Dox) attached via enzymatically degradable Gly-Phe-Leu-Gly spacer. (For structure, see Scheme 1.)

[1] Abbreviations: aq., aqueous; Boc, *tert*-butoxycarbonyl; Boc_2O, di-*tert*-butyl dicarbonate; Bz, benzyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCU, *N*,*N*′-dicyclohexylurea; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide; Dox, doxorubicin; FPLC, fast protein liquid chromatography; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; HPMA, *N*-(2-hydroxypropyl)methacrylamide; Me, methyl; M_n, number-average molecular weight; M_w, weight-average molecular weight; NAp, 4-nitroanilide group; NDA, naphthalene-2,3-dicarbaldehyde; Np, 4-nitrophenyl; OPA, phthaldialdehyde; OSu, *N*-succinimidyl; PEG, α-hydro-ω-hydroxypoly(oxyethylene); PEG-BSC, PEG bis(*N*-succinimidyl) carbonate; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Z, benzyloxycarbonyl.

EXPERIMENTAL SECTION

Doxorubicin hydrochloride was a kind gift from Dr. Suarato (Pharmitalia). PEG 2000, L-amino acids (if not stated otherwise), palladium catalyst, cathepsin B, and reagents for peptide synthesis were commercial products (Fluka AG, Switzerland). All other chemicals and solvents were of analytical grade. Solvents were purified and dried by standard procedures. The reagents were used without further purification. NMR spectra were recorded on a Bruker spectrometer (300 MHz, Switzerland). The block copolymers were characterized by size-exclusion chromatography on TSK columns 3000 and 4000 using 50% methanol as an eluent on FPLC system (Pharmacia, Sweden) calibrated with PEG standards. Amino acid analysis was performed by an amino acid analyzer (LDC Analytical) on a reversed-phase column Nucleosil 120-3 C_{18} (125 × 4 mm) (Macherey Nagel) with precolumn derivatization by NDA with 3-sulfanylpropanoic acid (excitation at 229 nm, emission at 490 nm) or OPA with 1-thio-β-D-glucose for determination of the optical purity of amino acids (λ_{exc} = 330 nm, λ_{em} = 420 nm) and using gradient elution from 35% to 100% of solvent B within 65 min and flow rate 0.5 mL/min (solvent A, 0.05 M sodium acetate buffer, pH 6.5; solvent B, 300 mL of 0.17 M sodium acetate and 700 mL of methanol) (*11*, *12*). Doxorubicin derivatives were analyzed by HPLC using Watrex reversed-phase column, Nucleosil C18, 250 × 4 mm, gradient MeOH-H_2O, 40–90% MeOH during 50 min, UV detector at 484 nm, fluorescence detector FluoroMonitor 4100, LDC Analytical (λ_{exc} = 490 nm, λ_{em} = 560 nm). Concentration of doxorubicin in the samples was determined by UV spectrophotometry at 484 nm (ϵ = 11 500 L mol^{-1} cm^{-1} in H_2O, 13 500 L mol^{-1} cm^{-1} in MeOH).

Preparation of Multiblock Copolymer poly[PEG-Glu-Lys-(-Glu)]. *PEG Bis(N-succinimidyl carbonate) (PEG-BSC) (1): Method A.* PEG 2000 (3.6 g, 1.8 mmol) azeotropically dried with toluene was dissolved in pyridine (20 mL) together with DMAP (88 mg, 0.72 mmol) and mixed with a solution of di-*N*-succinimidyl carbonate (1.8 g, 7.2 mmol) in acetonitrile (15 mL). The reaction mixture was protected from light at 25 °C for 12 h. The solvents were removed on a rotary evaporator, the residue was dissolved in ethyl acetate (50 mL, dried and distilled), at 50 °C, and the product was isolated by precipitation and filtration after addition of diethyl ether (50 mL) to the cooled ethyl acetate solution. This operation was repeated three times yielding 3.1 g of the active carbonate **1**.

Method B. PEG was activated by phosgene solution (20% in toluene) and the resulting chlorocarbonate derivative reacted with HOSu according to the literature (*13*).

Poly[PEG-GluLysGlu(OBz)]—Interfacial Polycondensation (2). Solution of PEG-BSC (1.3 g, 0.572 mmol) in dichloromethane (20 mL) was added to the mixture of tripeptide derivative **19** (0.516 g, 0.572 mmol) and sodium hydrogencarbonate (230 mg, 2.73 mmol) in water (20 mL) under vigorous stirring at 25 °C. The reaction mixture was acidified with 0.1 M HCl to pH 3 after 5 h of stirring. The organic layer was separated, washed with NaCl(aq) and dried over anhydrous Na_2SO_4. The drying agent was filtered, and the filtrate was concentrated to volume 10 mL. The precipitation of the polymer in diethyl ether failed (resulting in an oil layer). The solvent was removed under vacuum, and the residue was suspended in water (white emulsion) and freeze-dried. Yield: 1.1 g of polymer **2**.

Molecular weight of polymer strongly depends on reaction conditions (stirring speed, ratio of reactants, etc.). Usually M_w = 15000–30000.

Poly[PEG-GluLysGlu(OH)]—Hydrogenation (3). The polymer benzyl ester **2** (800 mg) was dissolved in ethanol (10 mL) with a drop of acetic acid and hydrogenated on a palladium catalyst (Pd/C, 10%) 3 h. The reaction mixture was bubbled with nitrogen, the catalyst was filtered, and ethanol was removed under vacuum. The residue was dissolved in water and freeze-dried yielding over 700 mg of hygroscopic polymer **3**.

Poly[PEG-GluLysGlu(ONp)] (**4**). The polymeric acid **3** (200 mg), 4-nitrophenol (100 mg), and DCC (140 mg) were dissolved in dichloromethane (2 mL) at 0 °C. A gel was formed almost immediately (probably due to the formation of acid anhydride linkages between COOH groups belonging to different polymer chains) but dissolved within 5 min. The reaction mixture was kept at 4 °C over the weekend. A drop of acetic acid was added and the precipitated DCU was filtered off 20 min later. The precipitation of the polymer in diethyl ether was unsuccessful (forming an oil). The solvents were evaporated and the polymer was purified (removal of 4-nitrophenol) by gel filtration (Sephadex LH20, dichloromethane). The polymer was isolated by freeze-drying from benzene resulting in a yellowish powder which "melts" when not kept under vacuum. Yield: 200 mg (90%) of the active ester **4**.

Preparation of Oligopeptide Derivatives. *HCl.Leu-Gly-OMe (5); HCl.Gly-Phe-OMe (6).* H-Leu-Gly-OH or H-Gly-Phe-OH were converted to the corresponding methyl esters by refluxing in dry MeOH with thionyl chloride (*14*). HCl·Gly-Phe-OMe was recrystallized from methanol-diethyl ether. HCl·Leu-Gly-OMe was obtained in crystalline form by trituration of an oily crude product with diethyl ether.

Tr-Gly-Phe-OMe (7). H-Gly-Phe-OMe·HCl (3.0 g, 11 mmol) was suspended in dry $CHCl_3$ (25 mL). Triethylamine (2.3 g, 22.7 mmol) was added, and the solid dissolved. Trityl chloride (3.1 g, 11.1 mmol) was added, and reaction mixture was left standing at 25 °C for 2 h. Chloroform was evaporated, and the oily residue was diluted with ethyl acetate (60 mL). Triethylamine hydrochloride precipitate was filtered off, and the filtrate was washed with water (2 × 20 mL) and dried over $MgSO_4$. Ethyl acetate was taken off, and the product was recrystallized from isopropyl alcohol-hexane. Yield was 4.5 g (9.4 mmol, 85%) of white crystalline product, mp 154–156 °C. TLC: R_f = 0.92 (silica gel, ethyl acetate).

Tr-Gly-Phe-OH (8). Tr-Gly-Phe-OMe (3.0 g, 6.27 mmol) was dissolved in dioxane (25 mL) and KOH(aq) (6.3 mL, 1 mol L^{-1}) was added. The reaction mixture was stirred at 45 °C for 1 h and then evaporated to dryness under reduced pressure. The crude product was dissolved in water (20 mL) and ethyl acetate was added followed by dilute acetic acid (20%). The free acid dipeptide derivative was taken up to ethyl acetate (3 × 20 mL), and collected organic extracts were dried over $MgSO_4$. White crystalline solid (2.8 g, 6.03 mmol, 96%) was obtained after filtration, concentration under vacuum, and trituration with hexane. TLC: R_f = 0.75 (silica gel, ethyl acetate).

Tr-Gly-DL-Phe-Leu-Gly-OMe (diastereomeric mixture, 9a, b). A solution of Tr-Gly-Phe-OH (2.8 g, 6.03 mmol) in THF (20 mL) was mixed with HCl·Leu-Gly-OMe (1.44 g, 6.08 mmol) and triethylamine (0.61 g, 6.03 mmol). A solution of DCC (1.5 g, 7.27 mmol) in THF (10 mL) was added with stirring at −5 °C. After 48 h at 4 °C, the

precipitated DCU was filtered off and the solution was concentrated under vacuum to give a viscous oily residue. The crude product was redissolved in ethyl acetate (30 mL), washed with water (3 × 20 mL), dried over $MgSO_4$ and finally purified by column chromatography (silica gel, ethyl acetate). The unreacted Tr-Gly-Phe-OMe was eluted first followed by the desired product. Yield after crystallization from ethyl acetate–hexane 2.65 g (4.08 mmol, 68%) of white crystalline solid, mp >90 °C (decomp.). TLC: R_f = 0.55 (silica gel, ethyl acetate) (L-Phe/D-Phe, 1:1).

Tr-Gly-L-Phe-L-Leu-Gly-OMe (9a). A solution of Tr-Gly-Phe-OH (0.89 g, 1.92 mmol), HCl·Leu-Gly-OMe (0.46 g, 1.93 mmol), HOBt (0.30 g, 1.93 mmol) in DMF (8 mL) was mixed with a solution of DCC (0.40 g, 1.93 mmol) in DMF (1 mL) at 0 °C followed by triethylamine (0.27 mL, 1.93 mmol). After 48 h at 4 °C, the precipitated DCU was filtered off and the solution was concentrated under vacuum to give a viscous oily residue. The crude product was redissolved in ethyl acetate (30 mL). The solution was washed with aq. solutions of $NaHCO_3$ and NaCl, dried over Na_2SO_4, filtered, and concentrated under vacuum. Yield after crystallization from ethyl acetate–hexane was 0.40 g (0.62 mmol, 33%) of pure L-Phe isomer, mp 172–175 °C. TLC: R_f = 0.55 (silica gel, ethyl acetate).

Tr-Gly-D-Phe-L-Leu-Gly-OMe (9b). was synthesized in the same way as the L-Phe isomer starting with D-phenylalanine.

Tr-Gly-DL-Phe-Leu-Gly-OH (diastereomeric mixture, 10a–b). Tr-Gly-DL-Phe-Leu-Gly-OMe (2.65 g, 4.08 mmol) was dissolved in dioxane (20 mL) and mixed with a KOH solution in dry methanol (4.2 mL, 1 mol L^{-1}). After 1 h at 50 °C, the solvent was taken off under reduced pressure, and the residue was redissolved in water (20 mL). The solution was covered with an ethyl acetate layer and acidified with dilute acetic acid (5%). The free acid was extracted with ethyl acetate (3 × 20 mL). The collected organic layers were washed with NaCl(aq), dried over $MgSO_4$, and filtered. The tetrapeptide derivative was crystallized from ethyl acetate-diethyl ether yielding 2.4 g (3.78 mmol, 93%) of white crystalline solid, mp 194–196 °C. (L-Phe/D-Phe, 1:1).

Tr-Gly-D-Phe-L-Leu-Gly-OH (**10b**) was obtained by repeated crystallization of the diastereomeric mixture from ethyl acetate- diethyl ether–hexane (D-isomer/L-isomer 95:5 after two crystallizations) as the less soluble isomer.

Tr-Gly-L-Phe-L-Leu-Gly-OH (**10a**) was isolated from the mother liquid of the above-described crystallization by precipitation with hexane (L/D, 90:10). The other method of the preparation of the tritylated tetrapeptide acids is based on the hydrolysis of the corresponding optically pure methyl esters as described for the diastereomeric mixture.

Tr-Gly-L-Phe-Leu-Gly-OSu (**11a**); *Tr-Gly-D-Phe-Leu-Gly-OSu* (**11b**). Tr-Gly-Phe-Leu-Gly-OH (0.5 g, 0.788 mmol), HOSu (0.1 g, 0.869 mmol), and DCC (0.2 g, 0.969 mmol) were dissolved in THF (8 mL) at 0 °C and kept at 4 °C overnight. Acetic acid (10 μL) was added and the precipitated DCU was filtered 30 min later. The solvent was removed under reduced pressure, and the crude product was crystallized from isopropyl alcohol-ether yielding 0.32 g (0.437 mmol, 55%) of the active ester. Mp 109–113 °C for L-Phe, 118–120 °C for D-Phe.

Tr-Gly-L-Phe-Leu-Gly-Dox (**12a**); *Tr-Gly-D-Phe-Leu-Gly-Dox* (**12b**). Dox·HCl (95 mg, 0.164 mmol) was dissolved in DMF (1.5 mL, freshly distilled from P_2O_5), and active ester **11a** or **11b** (120 mg, 0.164 mmol) in DMF (1.3 mL) was added followed by triethylamine (23 μL, 0.164 mmol) with stirring at 25 °C. The progress of the reaction was monitored by TLC (silica gel, chloroform–methanol–water, 120:20:1). Two diastereoisomers containing L-Phe and D-Phe residues (R_f = 0.66 and R_f = 0.63, respectively) were detected as red spots in visible light. After 24 h at room temperature, the solvent was evaporated to dryness, and the crude product was chromatographed on silica gel using chloroform–methanol (95:5) as an eluent. The fraction containing the diastereomeric mixture was evaporated to dryness and triturated with ether to yield 144 mg (0.124 mmol, 76%) of a red solid.

H-Gly-L-Phe-Leu-Gly-DOX·CH₃COOH (13a); H-Gly-D-Phe-Leu-Gly-DOX·CH₃COOH (13b). Trityl derivative **12a** or **12b** (140 mg, 0.121 mmol) was suspended in 75% acetic acid (2.8 mL, 35 mmol) and stirred at room temperature for 1 h. Any solid part was removed by centrifugation, and the supernatant was evaporated to dryness. The product was redissolved in water and freeze-dried yielding 65 mg (0.068 mmol, 57%) of the title acetate salt. The purity of the title compounds was determined by HPLC, indicating less than 5% of the undesired diastereoisomer, in accord with the amino acid analysis. The configuration of the amino acid residues was determined by HPLC of the hydrolyzed product.

Boc-Gly-Phe-OH (**14**) was prepared as described previously (*9*).

Boc-Gly-Phe-Leu-Gly-OMe (15). Boc-Gly-Phe-OH (2.92 g, 9.05 mmol), HCl·H-Leu-Gly-OMe (2.14 g, 9.05 mmol), HOBt (1.67 g, 10.9 mmol), and triethylamine (1.38 mL, 9.9 mmol) were dissolved in DMF (30 mL), and DCC (2.25 g, 10.9 mmol) was added with stirring at 0 °C. The progress of the reaction was checked by TLC (silica gel, ethyl acetate, R_f = 0.52). After 2 h at 0 °C and 20 h at 25 °C, the precipitated DCU was filtered off, the solvent was evaporated under vacuum and the oily residue was dissolved in ethyl acetate. The solution was filtered again and washed with $NaHCO_3$(aq), citric acid(aq), again $NaHCO_3$(aq), and NaCl(aq), and dried over Na_2SO_4. Then it was filtered and concentrated on rotavapor yielding 2.56 g (5.07 mmol, 56%) of white crystalline solid melting at 172–174 °C. Anal. ($C_{25}H_{38}N_4O_7$) C, H, N.

^{1}H NMR [$(CD_3)_2SO$]: δ 0.80 two d, 6H (CH_3-Leu); 1.31 s, 9H (Boc); 1.35–1.70 m, 3H ($CHCH_2$-Leu); 2.70 dd, 1H (CH_2-Phe); 2.95 dd, 1H (CH_2-Phe); 3.35–3.45 m, 2H (CH_2-N-Boc-Gly); 3.55 s, 3H (OCH_3); 3.75 t, 2H (Gly-OCH_3); 4.25 m, 1H (α-Leu); 4.50 m, 1H (α-Phe); 6.85 t, 1H (NH-Boc); 7.15 m, 5H (arom.); 7.80 d, 1H (NH); 8.10 d, 1H (NH); 8.20 t, 1H (NH).

^{13}C NMR [$(CD_3)_2SO$]: δ 19.71 (CH_3-Leu); 20.92 (CH_3-Leu); 22.04 (CH-Leu); 22.45 (CH_2-Leu); 26.16 (Boc); 31.35 (CH_2-Phe); 35.58 (CH_2-N-Boc-Gly); 41.19 (Gly-OCH_3); 48.82 (α-Leu); 49.64 (OCH_3); 51.52 (α-Phe); 76.07 (C-Boc); 124.19 (arom.); 125.97 (arom.); 127.27 (arom.); 135.55 (C-quart. arom.); 153.72 (OCONH); 167.12 (CO); 168.11 (CO); 168.60 (CO); 170.32 (CO).

H-Gly-L-Phe-Leu-Gly-OMe·TFA (**16a**). The corresponding N-Boc derivative **15** (0.5 g) was dissolved in neat TFA (5 mL). TFA was evaporated under vacuum after 1 h at 25 °C and a white crystalline product was obtained by trituration with diethyl ether in almost quantitative yield. Mp 160–162 °C.

Z-Gly-D-Phe-NHNH₂ (**17**) was prepared by hydrazinolysis of the corresponding methyl ester in methanol according to literature (*15*). The protected dipeptide hydrazide was recrystalized from methanol. Mp 104–105 °C. Anal. ($C_{19}H_{22}N_4O_4·CH_3OH$) calcd: C, 59.69; H, 6.52; N, 13.93. Found: C, 60.70; H, 6.36; N, 14.09.

Z-Gly-D-Phe-Leu-Gly-OMe (18). Z-Gly-D-Phe-$NHNH_2$ (1.7 g, 4.59 mmol) was dissolved in freshly distilled DMF (15 mL), the solution was cooled to −10 °C, and HCl (aq) (4 mol L^{-1}, 4.6 mL, 18.4 mmol) was added followed by $NaNO_2$ (aq) (397 mg in 0.7 mL, 5.75 mmol). Triethylamine (1.77 mL, 12.65 mmol) was added 5 min later, the reaction mixture was briefly dried with Na_2SO_4 and filtered directly to a solution of H-Leu-Gly-OCH_3·HCl (1.1 g, 4.59 mmol) and triethylamine (1.28 mL, 9.2 mmol) in 25 mL of DMF at −10 °C. The reaction was stirred for 2 h under cooling and then let warm to room temperature. The precipitated triethylamine hydrochloride was filtered off, the solvent was evaporated under vacuum, and the residue was dissolved in ethyl acetate (50 mL). The solution was washed with $NaHCO_3$(aq), $KHSO_4$, again $NaHCO_3$ (aq) and NaCl (aq). Then it was filtered and concentrated to crystallization to yield 1.19 g (2.2 mmol, 48%) of white crystalline solid, mp 138−139 °C. The content of the L-Phe isomer was <1% (by amino acid analysis). Anal. ($C_{28}H_{36}N_4O_7$) calcd: C, 62.21; H, 6.72; N, 10.37. Found: C, 61.78; H, 6.76; N, 9.99. TLC (silica gel, ethyl acetate, R_f = 0.39).

^{1}H NMR [$(CD_3)_2SO$]: δ 0.68 d, 3H (CH_3-Leu); 0.74 d, 3H (CH_3-Leu); 1.20−1.40 m, 3H ($CHCH_2$-Leu); 2.78 dd, 1H (CH_2-Phe); 2.87 dd, 1H (CH_2-Phe); 3.54 m, 2H (CH_2-N-Boc-Gly); 3.55 s, 3H (OCH_3); 3.75 d, 2H (CH_2-Gly-OMe); 4.18 m, 1H (α-Leu); 4.51 m, 1H (α-Phe); 4.96 s, 2H (CH_2-Ph); 7.17 m, 5H (arom.); 7.28 m, 5H (arom.); 7.30 t, 1H (NH-Gly-OMe); 8.05 d, 1H (NH-Phe); 8.22 d, 1H (NH-Leu); 8.23 t, 1H (OCONH).

^{13}C NMR [$(CD_3)_2SO$]: δ 19.32 (CH_3-Leu); 21.07 (CH_3-Leu); 21.79 (CH-Leu); 35.84 (CH_2-Phe); 38.50 (Gly-OCH_3); 38.60 (CH_2-Leu); 41.29 (CH_2-N-Boc-Gly); 48.72 (α-Leu); 49.63 (OCH_3); 52.12 (α-Phe); 63.45 (OCH_2Ph); 124.25 (arom., Phe-4); 125.66 (arom.); 125.76 (arom., Z-4); 126.00 (arom.); 126.32 (arom.); 127.19 (arom.); 135.01 (arom., Phe-1); 135.26 (arom., Z-1); 154.47 (OCONH); 166.95 (CO-Boc-Gly); 168.10 (COOMe); 168.66 (CO-Leu); 170.48 (CO-Phe).

H-Gly-D-Phe-Leu-Gly-OMe (16b). Z-Gly-D-Phe-Leu-Gly-OMe (270 mg, 0.5 mmol) was dissolved in ethanol (3 mL) with cyclohexa-1,4-diene (0.5 mL, 5 mmol). The solution was bubbled with nitrogen, palladium catalyst (10% on active carbon) was added, and the reaction mixture was stirred under nitrogen overnight. The catalyst was filtered off, and the filtrate was evaporated to dryness. The amorphous residue was triturated with diethyl ether to yield 110 mg (0.27 mmol, 54%) of white crystalline product with mp 98−102 °C. Anal. ($C_{20}H_{30}N_4O_5$) calcd: C, 59.09; H, 7.45; N, 13.78. Found: C, 57.91; H, 7.32; N, 13.15.

^{1}H NMR [$(CD_3)_2SO$]: δ 0.68 d, 3H (CH_3-Leu); 0.74 d, 3H (CH_3-Leu); 1.20−1.40 m, 3H ($CHCH_2$-Leu); 2.83 m, 2H (CH_2-Phe); 3.35 m, 2H (CH_2-Gly); 3.55 s, 3H (OCH_3); 3.75 m, 2H (CH_2-Gly-OMe); 4.17 m, 1H (α-Leu); 4.54 m, 1H (α-Phe); 7.16 m, 5H (arom.); 8.01 br, 1H (NH-Leu); 8.24 d, 1H (NH-Phe); 8.29 t, 1H (NH-Gly-OMe).

Preparation of Diamine Linker "GluLysGlu". *N,N'-Bis(-benzyl glutamyl)lysine benzyl ester bis(trifluoroacetate)* (**19**) was prepared as described previously (*8*).

Preparation of Polymer Conjugates of DOX. *Poly[PEG-GluLysGlu(Gly-Phe-Leu-Gly-OMe)] (20a and 20b; L-Phe and D-Phe derivatives, respectively).* Polymer acid **3** (0.5 g, 6 × 10^{-4} mol of COOH groups), tetrapeptide derivative **16a** (100 mg, 2 × 10^{-4} mol) or **16b** (81 mg, 2 × 10^{-4} mol), HOBt (153 mg, 1 mmol), triethylamine (140 μL, 1 mmol), and DCC (206 mg, 1 mmol) were dissolved in DMF (3 mL) at 0 °C and left at 4 °C over weekend. Dilute acetic acid (50%, 0.2 mL) was added to the reaction, and the precipitated DCU was filtered off 20 min later. DMF was removed under reduced pressure, and the residue was dissolved in chloroform. The organic solution was washed with NaCl (pH 2 adjusted with HCl), dried over Na_2SO_4, filtered, evaporated to dryness and the residue was chromatographed on Sephadex LH 60 in methanol (RI detector). The high-molecular-weight fraction was collected and used for the subsequent reaction. Yield: 254 mg of white polymer methyl ester **20a** or **20b**. Coupling efficacy calculated from the results of amino acid analysis was 75% of the starting tetrapeptide derivative.

Poly[PEG-GluLysGlu(Gly-Phe-Leu-Gly-NHNH$_2$)] (21a and 21b; L-Phe and D-Phe derivatives, respectively). The polymer methyl ester **20a** or **20b** (0.25 g, 86 × 10^{-6} mol of COOMe) and hydrazine hydrate (0.1 mL, 2.86 × 10^{-3} mol) were dissolved in absolute methanol (2.5 mL). After 2 days at 25 °C, the solvent was taken off under reduced pressure, and the residue was freeze-dried from water solution yielding 240 mg of the polymer hydrazide **21a** or **21b**.

Poly[PEG-GluLysGlu(Gly-Phe-Leu-Gly-DOX)] (22a and 22b; L-Phe and D-Phe derivatives, respectively; polymer 22 prepared from 1:1 diastereomeric mixture of 13a and 13b). Method A. Polymer hydrazide **21a** or **21b** (0.1 g, 36 × 10^{-6} mol of −$NHNH_2$) and diluted HCl (4 M, 60 μL) were dissolved in DMF (0.7 mL), and $NaNO_2$ (aq) (5 M, 40 μL) with 0.2 mL of DMF was added at 0 °C with stirring. Triethylamine (50 μL, 0.36 mmol) and anhydrous Na_2SO_4 (200 mg) were added about 5 min later followed by Dox·HCl (20 mg, 35 μmol) again after 5 min. The reaction mixture was kept 30 min at 0 °C with stirring, then placed to 4 °C overnight. Any insoluble material was removed by filtration, the solvent was evaporated under reduced pressure, and the residue was purified by gel filtration. After chromatography on PD10 columns (Pharmacia, Sephadex G25) in water, some low-molecular-weight derivatives of doxorubicin were found by analytical HPLC in MeOH, which were completely removed by chromatography on Sephadex LH20 in MeOH. Yield: 98 mg of very hygroscopic red polymer **22a** or **22b**. Content of polymer-bound doxorubicin was 6.0%w/w (10 μmol, 30% of starting Dox).

Method B. Polymeric acid **3** (80 mg, 87 × 10^{-6} mol of COOH groups), tetrapeptide derivative of Dox **13a** or **13b** (28 mg, 29 × 10^{-6} mol), HOBt (44 mg, 0.29 mmol), triethylamine (4 μL, 29 × 10^{-6} mol), and DCC (60 mg, 0.29 mmol) were dissolved in DMF (2.5 mL) at 0 °C and left at 4 °C overnight. Dilute acetic acid (50%, 0.1 mL) was added to the reaction and the precipitated DCU was filtered off 2 h later. DMF was removed under reduced pressure, the residue was dissolved in MeOH (5 mL), and the remaining DCU was filtered off. The residue was chromatographed on Sephadex LH20 in MeOH. The high-molecular-weight fraction was collected and freeze-dried yielding 82 mg of the polymer **22a**, **22b**, or **22** with 6.5% w/w of Dox. M_w = 29 000 and M_w/M_n = 3.3 were determined by FPLC. The amino acid analysis of the hydrolyzed product **22** found molar ratio Gly:L-Phe:D-Phe:L-Leu = 2:0.5:0.5:1. Polymer **22** was used for the in vitro and in vivo experiments described below.

Enzymatic Hydrolysis of the Polymer Substrate (Figure 1). Enzymatic degradation of the polymer **22** (prepared by method B) by cathepsin B was performed according to the previously published procedure (*9*) (0.05 M phosphate buffer, 0.001 M EDTA, pH 6.0, 37 °C, 0.005 M glutathione), increasing the concentration of the active sites of cathepsin B to 4 × 10^{-7} M due to the slow rate of the enzymatic reaction. Both the release of Dox and the

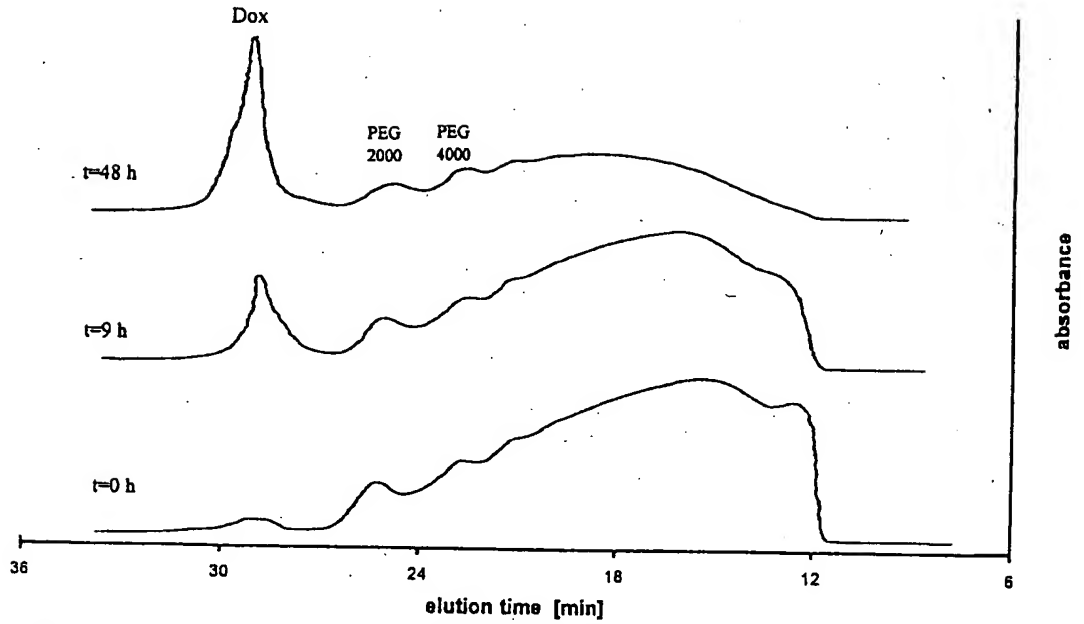


Figure 1. GPC records of degradation products of polymer **22** incubated with cathepsin B (0, 9, and 48 h after the addition of the enzyme) [E] = 4×10^{-7} M, [Dox] = 0.001 M, [polymer] = 21 mg/mL, 0.05 M phosphate buffer, pH 6.0, 37 °C, column TSK 3000, 50% MeOH, 0.1% TFA, UV 480 nm.

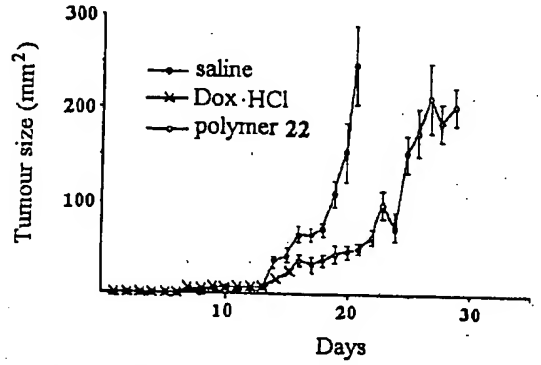


Figure 2. Anti-cancer activity of free and polymer-bound doxorubicin 5 BALB/C female mice were injected i.v. on 3 consecutive days (9, 10, and 11). One dose was 100 μL of a drug solution in saline of concentration 1 mg/mL, i.e., 5 mg/kg calculated for content of Dox·HCl. The mice were bearing s.c. murine colorectal carcinoma C26 injected 8 days earlier.

degradation of the block copolymer were monitored by FPLC (column TSK 3000, 50% MeOH, 0.1% TFA, UV detector, 484 nm, RI detector).

In Vivo Evaluation (Figure 2). Polymer **22** (prepared by method B), Dox·HCl, and physiological saline were injected each to groups of five BALB/C female mice i.v. on three consecutive days (9, 10, and 11). One dose was 100 μL of the drug solution in saline of concentration 1 mg/mL, i.e., equivalent to 5 mg/kg of Dox·HCl. The mice were bearing s.c. murine colorectal carcinoma C26 injected 8 days earlier. Solid tumors were just palpable at the start of the drug administration.

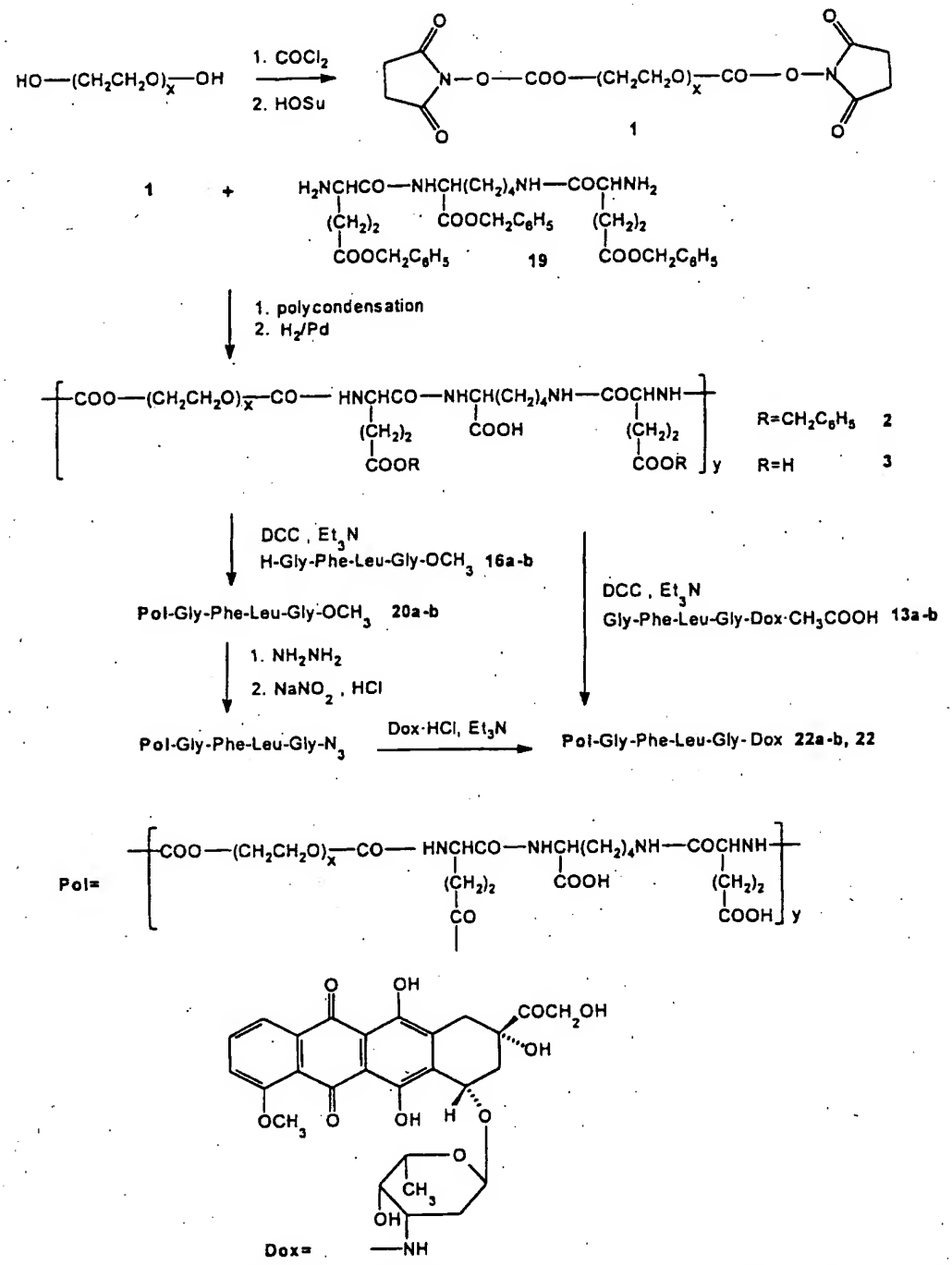
RESULTS AND DISCUSSION

Synthesis of the Block Copolymer. The alternating copolymer of PEG and the oligopeptide-based diamine linker Glu-Lys(-Glu) was prepared by interfacial polycondensation (dichloromethane-water) of the activated bifunctional PEG 2000 (PEG-BSC) **1** and oligopeptide **19** protected by benzyl ester moieties (Scheme 2). The polycondensation was carried out according to the method of Zalipsky and co-workers (*7*, *16*) who described synthesis of analogous copolymer based on ethyl lysinate and PEG. Nevertheless, the molecular weight of the resulting copolymers (M_w = 50 000) prepared with our tripeptide diamine linker was lower than the molecular weight of those described in the literature (M_w = 100 000). That is quite obvious if all the many factors influencing the course of the polycondensation reaction are taken into account. The most important of them are the solubility of reacting components in both phases, the yield of substitution of PEG derivative, and its hydrolytic stability, reactivity of the functional groups, presence of impurities, etc. For GPC, see Figure 1 (t = 0); no significant change was observed before and after drug attachment.

The block copolymer **2** was hydrogenated on palladium catalyst to remove the protecting benzyl ester groups, and the resulting copolymer with three carboxyls in each repeating block was used for the attachment of the oligopeptide spacer and doxorubicin. FPLC of the polymers confirmed that there were no changes in molecular weight and its distribution during the modification reactions (deprotection, binding of spacer and drug, etc.). The polycarboxylic acid **3** can be further converted to the corresponding active 4-nitrophenyl ester **4**. This reaction step is not necessary for subsequent coupling with spacer and drug but results in a convenient precursor for further modification with a targeting moiety (antibody or its fragments, lectins, etc.) in aq. media.

Attachment of the Drug to the Polymer Carrier. The covalent attachment of doxorubicin to the polymer carrier was performed by two different synthetic routes, both leading to the same final structure with the amide bond between the drug moiety and the tetrapeptide spacer. The first method involves quite elaborate and expensive synthesis of the Gly-Phe-Leu-Gly-Dox derivative and its conjugation with the polymer carrier, the other is based on the azide procedure commonly used in peptide synthesis. In the latter case, free doxorubicin can

Scheme 2



be directly coupled with the reactive copolymer-tetrapeptide precursor.

The first route leading to the suggested polymer drug consists of synthesis of the tetrapeptide derivative H-Gly-Phe-Leu-Gly-Dox and its covalent binding to the carboxylic groups of the polymer carrier via amide bond. The synthesis started from the methyl esters H-Leu-Gly-OMe and H-Gly-Phe-OMe. The latter reacted with triphenylmethyl (trityl) chloride yielding the corresponding N-trityl derivative, which was converted to the free acid by alkaline hydrolysis. Tr-Gly-Phe-OH was coupled with H-Leu-Gly-OMe in the presence of DCC to yield protected tetrapeptide Tr-Gly-Phe-Leu-Gly-OMe. Most probably, racemization of the phenylalanine residue occurred in this step if no HOBt was added to the reaction as determined by amino acid analysis. The methyl ester was hydrolyzed to the free carboxylic acid, which was converted to the active succinimidyl ester used for the acylation of the primary amino group of doxorubicin. The resulting tetrapeptide derivative Tr-Gly-Phe-Leu-Gly-DOX gave two spots on TLC plate when a diastereomeric mixture was used for the acylation. The two diastereoisomers of the tritylated tetrapeptide-Dox derivative could be partially separated by column chromatography on silica gel. The protecting trityl group was removed by reaction with dilute acetic acid, and the desired H-Gly-Phe-Leu-Gly-Dox was bound to the pendant carboxylic groups (Glu and Lys residues) of the polymer carrier.

The described synthetic route requires a quite difficult and time-consuming preparation of the tetrapeptide

derivative of doxorubicin. Therefore, we have tried another strategy based on the well-known azide procedure. Methyl ester of the tetrapeptide was prepared and coupled with the free carboxylic groups of the block copolymer precursor. The resulting polymer methyl ester was converted to hydrazide by reaction with hydrazine, and the hydrazide was transformed to the active polymer azide by reaction of the hydrazide moiety with nitrous acid. The azide was then used in the reaction of the polymer with the primary amino group of doxorubicin. The major advantage of this synthetic pathway is that we work with the expensive drug only in the last reaction step compared with the three steps of the other method.

Biological evaluation of poly(HPMA)-Dox conjugates have shown the highest anti-tumor activity (both in vitro and in vivo experiments) when the D/L ratio of Phe residues in the tetrapeptide spacer was 1:1. (Diastereomeric mixture of the tetrapeptide precursor was used in the synthesis.) Although this interesting effect has not been yet fully explained, the use of the diastereomeric mixture in the synthesis of doxorubicin conjugates with PEG block copolymers is quite acceptable. The influence of the Phe configuration on the biological activity of the PEG-based polymer anticancer drug is being investigated at present and the corresponding polymer conjugates with doxorubicin are under biological evaluation. We have found that the biodegradability of the spacer and the drug release is a prerequisite for the biological activity in vivo, but the higher rate of enzymatic release (L-Phe > DL-Phe > D-Phe in the tetrapeptide spacer) of the drug from the polymer carrier in vitro does not necessarily mean a better anti-cancer activity in vivo as we discovered (*17*) using the poly(HPMA)-based doxorubicin conjugates.

Enzymatic Degradation of the Polymer Drug. It was reported (*6*) that high-molecular weight polymers are predominantly accumulated in solid tumors (EPR effect). The degradability of the polymer drug carrier is a prerequisite for its therapeutic application if the polymer molecular weight exceeds the renal threshold (approx. 50 000). The incubation of the polymer substrate **22** with lysosomal enzyme cathepsin B (Figure 1) resulted both in the splitting of the diamine linkages between the PEG blocks and in the release of doxorubicin. Both RI and UV detectors gave almost the same GPC profiles after injection of the polymer **22** incubated with cathepsin B. The chromatograms obtained from both detectors were completely superimposable except for the low-molecular-weight region where only UV detector at 484 nm indicated the amount of the drug (or its low-molecular-weight derivatives) released by the enzymatic reaction. (The response of the RI detector in this region is overlapped by solvent signal, so-called "buffer peak"). No degradation was observed in the case of a control sample incubated in physiological saline under the same conditions. About 30% of the total amount of the drug was released within 48 h of incubation with the enzyme. Only about 10% of the starting PEG 2000 was detected after that time, but the molecular weight of the polymer changed dramatically, indicating the degradation of the block copolymer both from the ends and in the middle of the chain.

Cathepsin B was chosen as a representative of lysosomal enzymes, the specificity of which is very close to that one of tritosomes (mixture of rat-liver lysosomal enzymes). As we found in our previous studies (*18*), glycine derivative of doxorubicin (60%) was released together with the free drug (40%) during the incubation with cathepsin B, but it was further degraded to free doxorubicin when tritosomes were used instead of cathepsin B. The rate of the drug release seems to be higher than the rate of the polymer carrier degradation. That is an important requirement for the anti-tumor activity of the whole system in vivo. The polymer drug–conjugate suggested in our work meets that well.

In Vivo Evaluation of Anti-Cancer Activity. Preliminary results of anti-cancer efficacy of our new polymer drug **22** (administered i.v.) in murine colorectal carcinoma C26 have shown quite significant inhibition of the tumor growth compared with the control group treated only with physiological solution (Figure 2). Unmodified doxorubicin hydrochloride was administered to another group of animals at the same dose as in the group treated with the polymer–Dox conjugate (dose converted to Dox·HCl). The mice treated with the free cytostatic drug had to be put down about 5 days after the drug administration due to the severe acute toxicity. A significant loss of body weight was observed in that group. On the other hand, the polymer-bound doxorubicin has shown no signs of toxicity at all (no decrease of body weight, good performance of the animals). The colorectal carcinoma chosen for the experiment generally exhibits a very low sensitivity to any common chemotherapy. Although we are aware of the fact that we are presenting only preliminary data obtained with a limited amount of the target compound **22**, which must be repeated in a more detailed study, the absence of toxicity of the polymer drug in particular and the inhibition of the tumor growth encouraged us to publish these promising results.

The polymer system described in this work consists only of the nontargeted polymer carrier and the drug moiety. We know that analogous nontargeted poly(HPMA)-based conjugates with doxorubicin exhibit much lower anti-tumor activity both in vitro and in vivo compared with the antibody-targeted ones (*19*, *20*). Therefore, we recently tried to prepare corresponding conjugates with antibody starting with the active ester **4**. Biological activity of the resulting biodegradable PEG block copolymers targeted with antibodies specifically interacting with receptors of the target cancer cells is being tested at present. On the basis of the results obtained with the HPMA-based polymer drugs, we expect the anti-cancer activity of such targeted biodegradable copolymers to be significantly higher than that of polymer **22**.

CONCLUSIONS

Two different synthetic routes for preparation of a water-soluble high-molecular-weight polymer drug carrier based on PEG block copolymer have been developed. The first method involves a quite elaborate and expensive synthesis of the Gly-Phe-Leu-Gly-Dox derivative and its conjugation with the polymer carrier, the other is based on the azide procedure used in peptide synthesis. In the latter case, free doxorubicin can be directly coupled with the active copolymer–tetrapeptide precursor. The degradability of the proposed polymer carrier is a prerequisite for successful excretion by glomerular filtration of high-molecular-weight polymer drugs after release of the active substance. Preliminary results of biological evaluation of the polymer–Dox in vivo have shown a lower toxicity and higher anti-tumor activity compared with free Dox using murine C26 colorectal carcinoma as a model tumor (this paper). Full assessment of advantages of the biodegradable carrier system compared with the nondegradable poly(HPMA) carriers would require de-

tailed evaluation of their activity with various tumor models. Our latest results show significantly improved anti-cancer efficacy compared with poly(HPMA)–Dox conjugate in mice bearing murine EL-4 lymphoma.

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